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Lipocalin 2 and the pathophysiology of Alzheimer's disease

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Chapter 1

General introduction

General introduction

Our abilities to think, perceive, feel, create and store and retrieve memories, make decisions, plan and carry out actions and display behavior depend on the functioning of our brains. This proper functioning of the brain relies on the health of the brain cells that inhabit our brain. The brain consists of different types of brain cells, including amongst others: neurons, astrocytes, microglia and oligodendrocytes.

Neurons are nerve cells, which communicate with each other via electrical signals. A neuron can send electrical signals along its axon, which is a long extension that protrudes from the neuron's cell body, until the signals reach the end of the axon. Here, electrical signals are often converted into chemical signals known as neurotransmitters. These neurotransmitters travel through the space that connects neurons, called the synaptic cleft. Upon arrival at the target neuron, the chemical signals are converted back into electrical signals, after which the targeted neuron itself may send out electrical signals to other connected neurons, and so on. Each neuron has communication points (called synapses) with many other neurons, and healthy neuronal communication within and between different brain regions is essential for the functioning of the brain.

Besides neurons, the brain consists of cell types including microglia, astrocytes and oligodendrocytes, which are collectively called glia. Glia – Greek for glue – were once believed to simply be the glue that holds the brain together. However, it is clear now that glia execute many more crucial functions in the brain. Microglia are known as the immune cells of the brain. Microglia scan the brain for signs of danger and damage, and become activated when they encounter pathogens or damaged brain cells. In this activated immune state they will try to remove these pathogens and damaged brain cells (by 'eating' them, a process called phagocytosis), to protect the brain and to promote repair of damaged brain tissue. In addition, microglia can modulate connections between neurons, by removing synapses. Astrocytes exert immune functions as well, and can also modulate synapses. Moreover, astrocytes provide nutrients to neurons, and protect neurons against overstimulation by taking up excess neurotransmitters from the synaptic cleft. Furthermore, astrocytes are an important component of the blood-brain barrier. The blood-brain barrier protects the brain from potential damaging factors and cells present in the blood by preventing their entry into the brain, while allowing oxygen and nutrients to pass into the brain. Also, astrocytes can influence the constriction and dilation of blood vessels, and thereby affect blood flow in the brain. Oligodendrocytes wrap the axons of neurons with a fatty substance called myelin, thereby insulating axons and improving fast conduction of electrical signals along axons. Due to the white appearance of myelin, brain regions that mainly contain myelinated axons are called white matter (as opposed to grey matter, which contains neuronal cell bodies).

Healthy neurons and glia, and healthy communication between them, are crucial to maintain brain health. Unfortunately, the functioning of neurons and glia can become disturbed, as evidenced by different brain diseases, such as Alzheimer's disease (AD).

Alzheimer's disease

AD is the most common cause of dementia, resulting in memory loss and problems with performing normal activities of daily living, which become increasingly worse with time [1,2]. In addition, AD is often accompanied by behavioral changes and neuropsychiatric problems, such as agitation, anxiety and depression [3].

AD accounts for 60-75% of all cases of dementia, which worldwide is estimated to affect 5-11% of people over the age of 60, and 20-50% of people over the age of 85 years [4–14]. Since the risk of dementia increases with age, and life expectancy continues to increase globally, the number of dementia patients will only grow further in the coming decades. Currently, worldwide around 50 million people have dementia, and this number is expected to triple to around 150 million in 2050 [14]. AD (and other types of dementia) comes with a very high social burden for family and caregivers of AD patients, as well as with great economic costs. Many past and present investigations have/are focused on finding possible treatments for AD, in the hope to slow down, halt or even prevent this awful disease. Unfortunately, besides a few drugs that temporarily improve AD symptoms, no effective treatments have been identified so far. The fact that no effective treatments for AD are present yet, may depend for an important part on the incomplete understanding of AD.

What goes wrong in the Alzheimer brain?

AD pathology causes brain damage in the brains of AD patients. AD pathology starts in brain regions important for learning and memory, such as the brain region named the hippocampus. With time, AD pathology worsens and spreads out further throughout the brain, resulting in worsening of cognitive and behavioral problems over time. How does AD pathology start, and how does AD pathology cause brain damage? Despite years of intensive research, the pathological processes that underlie the brain damage in AD, and the causes that initiate these pathological processes, are not fully understood yet.

It is known that the AD brain is characterized by different pathological hallmarks. First of all, as was already reported in the first description of AD by Dr. Alois Alzheimer in 1906, the brain of AD patients is marked by abnormal accumulations of certain proteins, present between brain cells and within neurons [15]. The protein accumulations between brain cells were later found to consist of aggregated amyloid- β (A β) protein, and were named plaques. The protein accumulations within neurons were identified as aggregates of hyperphosphorylated tau protein, and were named tangles. Secondly, it has become clear that chronic inflammation in the brain (neuroinflammation) is an important hallmark of AD [16,17]. This chronic neuroinflammation is mediated by chronically activated microglia and astrocytes, and is apparent from for example the altered shape of these cells when they are activated as well as from increased levels of inflammatory factors, which are released (secreted) by these cells. Next to aggregation of A β and tau and chronic neuroinflammation, brain regions affected by AD are marked by multiple other pathological changes, including accumulation of iron, disturbed energy metabolism, dysregulated neurotransmitter

metabolism, disturbed blood flow, disruption of the blood-brain barrier, white matter damage, synaptic damage and neuronal loss [18–26].

All of these pathological processes may be important contributors to the brain damage and symptoms that arise in AD. For example, A β aggregates (especially smaller aggregates, named oligomers) are known to promote all other above-mentioned pathological processes [27,28]. In addition, it has become clear that also chronic neuroinflammation has widespread effects [16]. Namely, when microglia and astrocytes become chronically activated, they may lose some of their protective functions such as removing damaged or dead cells and A β aggregates. Instead, chronically activated microglia and astrocytes may promote brain damage. For example, chronically activated microglia and astrocytes can continuously secrete pro-inflammatory and other toxic factors, which may amongst others disturb iron and energy metabolism, and provoke synaptic damage and cell death [16,29–31]. Interestingly, many of the pathological processes in AD influence each other, and promote each other's progression. For example, A β aggregation, iron accumulation and chronic neuroinflammation seem to stimulate each other's continuation, thereby together driving the progression of AD pathology [16,24,32,33].

What are the causes of Alzheimer's disease?

What initiates these pathological processes in AD, as described above? A small part (~1%) of AD cases is directly caused by genetic mutations [34]. In this familial form of AD, patients carry mutations in genes that are involved in the production of A β . This results in an overproduction of A β , and early onset of AD (before 65 years of age). However, for the vast majority (~99%) of AD cases, the underlying causes are far less clear [35,36]. This main type of AD is called sporadic AD, and usually starts later in life (after the age of 65). The major risk factor for sporadic AD is increasing age. In addition, certain genetic variants have been linked with an increased risk to develop sporadic AD [37]. Furthermore, risk factors for sporadic AD include for example: unhealthy lifestyle (e.g. smoking, physical inactivity and stress), various diseases (e.g. cardiovascular disease, diabetes, obesity and depression), certain injuries (e.g. surgery and traumatic brain injury), specific environmental factors (e.g. certain metals, air pollutants and pesticides), and infections (including certain bacterial, viral and fungal infections) [38–41]. Interestingly, many of the risk factors for sporadic AD, including aging, genetic variants, unhealthy lifestyle, injury and disease, have been linked with A β accumulation and chronic neuroinflammation, thereby supporting the importance of A β aggregation and chronic inflammation in the development and progression of AD [37,42–44].

Taken together, different risk factors for sporadic AD have been identified, and many pathological processes have been recognized to arise in the AD brain, which may all contribute to the brain damage in and symptoms of AD (also see Fig. 1). As such, it appears that sporadic AD is likely caused by a complex combination of aging and genetic, lifestyle and environmental factors, which may together provoke different AD-related pathological processes in the brain, such as A β aggregation and chronic neuroinflammation. However,

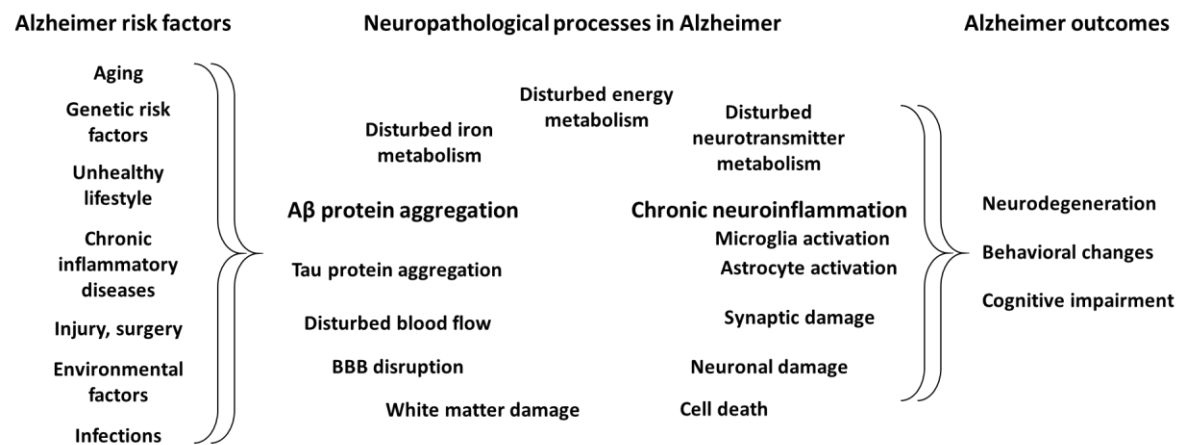


Fig. 1 Risk factors for sporadic AD, and pathological processes known to be involved in AD pathology, resulting in AD neurodegeneration and dementia.

many questions regarding the causes and pathophysiology of sporadic AD remain, including the molecular mechanisms that are involved. For example, for many pathological processes in AD it is not fully understood yet via what exact molecular mechanisms they are initiated. Moreover, the mechanisms that underlie the toxic effects of these pathological processes are not completely clear yet. In order to develop effective treatments for AD, it is essential to gain more insight into the molecular mechanisms and factors that are involved in the development and progression of AD pathology. Since the importance of chronic (neuro)inflammation in AD and risk factors of AD is increasingly recognized, a better understanding of (neuro)inflammatory processes and involved inflammatory factors may be key to elucidate how AD starts and progresses, and how it may be stopped or prevented.

Recently, a protein called Lipocalin 2 (Lcn2) was identified as a potential important new player in AD. As described below, Lcn2 may significantly affect (neuro)inflammatory processes. Moreover, Lcn2 may affect several other processes, including iron and energy metabolism, and cell death/survival.

Lipocalin 2 (Lcn2)

Lcn2 is a member of the lipocalin family of proteins, which binds and transports small hydrophobic factors [45]. Lcn2 has been implicated to play a role in different physiological and pathophysiological processes. One of the most well-known functions of Lcn2 is its role as an acute-phase protein in the defense against certain bacteria [46,47]. Bacteria require iron for their growth, and secrete small iron-binding factors called siderophores to collect iron. Lcn2 however can interfere with this bacterial iron uptake, by binding to bacterial siderophores and thereby preventing their delivery to bacteria. As such, Lcn2 exerts important antibacterial effects by hijacking bacterial iron acquisition. Interestingly, more recently it has become clear that Lcn2 may also be involved in normal physiological iron metabolism, by binding mammalian siderophores [46,47]. Besides its functions in antibacterial defense and mammalian iron metabolism, Lcn2 is known to play a role in

various other processes, including inflammation, cell migration, energy metabolism and cell death/survival signaling (Fig. 2a) [46,47].

Under healthy conditions, the gene expression and protein levels of Lcn2 are low. Interestingly, Lcn2 levels in blood were found to gradually increase with advancing age, which may reflect the chronic low-grade inflammation that accompanies aging [48]. In addition, local and circulating protein levels of Lcn2 are greatly increased upon pathogenic threats and different kinds of injury, as well as in a wide range of diseases [47]. Lcn2 was found to significantly influence the severity, development and/or progression of several of these infections, injuries and diseases, such as chronic kidney disease and different types of cancer [45–47]. The role of Lcn2 in these conditions may rely for example on its involvement in inflammation, iron metabolism and cell death/survival signaling. Interestingly, contradicting effects have been reported for Lcn2, including e.g. anti- and pro-inflammatory, and anti- and pro-cell survival effects [46,47]. These contradictory effects of Lcn2 may depend on many factors, such as the precise disease condition and the cell types and tissues that are involved.

A role for Lcn2 in Alzheimer's disease?

The role of Lcn2 in the healthy, injured and diseased brain has only more recently been explored. As in most tissues elsewhere in the body, the expression of Lcn2 in the brain is low under healthy conditions [47]. Also, Lcn2 expression in the brain was reported to gradually increase with advancing age in mice, which may correspond with the chronic low-grade (neuro)inflammatory state that arises in the body and brain with increasing age [49]. Besides in aging, elevated peripheral and brain levels of Lcn2 were also observed in other risk factor conditions of age-related brain diseases, such as obesity, cardiovascular disease and depression [50–53]. Moreover, brain Lcn2 levels were found to increase manifold upon inflammatory stimulation and brain injury, as well as in various brain conditions such as multiple sclerosis and stroke [47,54]. The effects of Lcn2 have been studied in several cell culture and animal models of these brain conditions. Although a few studies noted a beneficial effect of Lcn2, most studies found that increased Lcn2 levels significantly aggravated different neuropathological processes. For example, Lcn2 was reported to promote pro-inflammatory activity of microglia and astrocytes, to provoke iron accumulation in the brain, to aggravate disruption of the blood-brain barrier and white matter damage, and to stimulate neuronal cell death. Furthermore, Lcn2 has been suggested to be involved in disease-related behavioral and cognitive changes [47,54].

It may be hypothesized that Lcn2 may exert similar effects in the AD brain, and as such could contribute significantly to the development and progression of AD (Fig. 2b). A recent study has shown that Lcn2 protein levels are increased in the hippocampus, in human post-mortem brain tissue of AD patients [55]. Moreover, cell culture studies showed that A β induces Lcn2 production in cultured astrocytes, and that Lcn2 sensitizes neurons and astrocytes to A β -induced cell death [55,56]. However, most of these findings have not yet been replicated. Moreover, the effects of Lcn2 have not been investigated yet *in vivo* in animal models of AD. Therefore, further research is essential to gain more insight into the

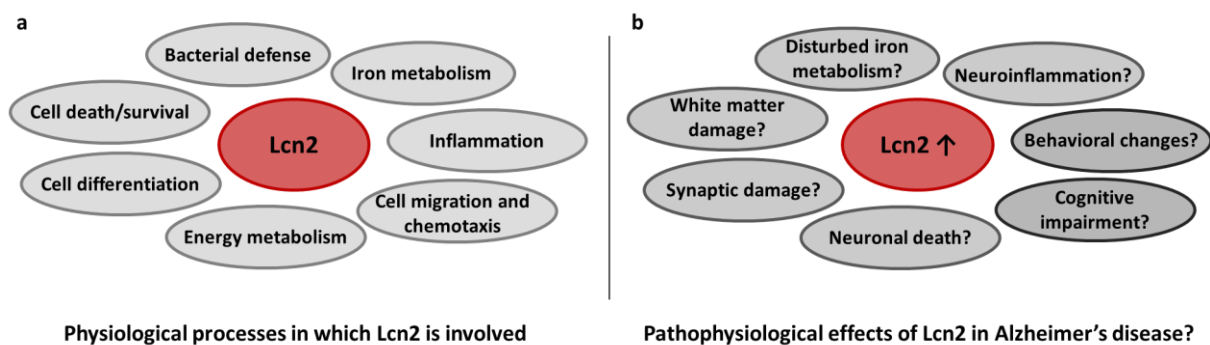


Fig. 2 (a) Physiological processes in which Lcn2 is known to play a role, and **(b)** AD-related (patho)physiological processes to which Lcn2 might contribute. Also see chapter 6 of this thesis and reviews [47,54] for further explanation.

potentially important role of Lcn2 in AD. More research is also needed to clarify whether Lcn2 may be a promising therapeutic target or diagnostic marker for AD.

Aims and outline of the thesis

In this thesis, we aim to gain a better understanding of the role of Lcn2 in AD. First, in **chapter 2**, we studied Lcn2 protein levels in blood, cerebrospinal fluid and post-mortem brain tissue of human AD patients and healthy age-matched persons. This study confirms that Lcn2 levels are significantly increased in the AD brain as compared to the healthy aged brain, in multiple brain regions. Furthermore, we found that co-existing depression in AD was related to significantly altered Lcn2 protein levels in different brain regions. After confirming increased Lcn2 levels in the brains of human AD patients, we in **chapter 3** studied the role of Lcn2 in the J20 mouse model of AD. J20 mice overexpress two mutated human genes that in humans are known to cause familial AD. Indeed, J20 mice develop AD-like pathological characteristics, including A β plaque pathology, neuroinflammation and cognitive impairment. As expected, we found that Lcn2 protein levels are increased in the brain of J20 AD mice, as compared to normal (wildtype) mice. We then compared J20 mice with J20 mice that are deficient in Lcn2. We found that J20 and Lcn2-deficient J20 mice show equally severe memory impairment, A β plaque load, and activation of microglia and astrocytes. Interestingly, Lcn2-deficient J20 mice showed less severe brain iron accumulation, as compared to J20 mice. In **chapter 4** we aimed to gain a better understanding of the regulation of Lcn2 production, and to identify potential inhibitors of Lcn2 overproduction, in cultured mouse astrocytes. We confirmed that A β induces a strong increase in Lcn2 protein production in astrocytes. Moreover, we show that iron chelators can inhibit this A β -induced Lcn2 production, and that A β may directly disturb iron metabolism in cultured astrocytes. In **chapter 5**, we explored the potential suitability of Lcn2 as a biomarker in age-related diseases, by assessing whether Lcn2 protein levels in blood and cerebrospinal fluid remain stable throughout the day in healthy elderly people. Indeed, Lcn2 levels did not fluctuate significantly during the day, which is a favorable characteristic for a biomarker. In **chapter 6** we review the current knowledge regarding the potential role of Lcn2 in age-related brain

diseases including AD, Parkinson's disease (PD) and vascular dementia (VaD). In addition, we discuss the role of Lcn2 in risk factor conditions for these disorders, and explore the possibility that Lcn2 is an inflammatory link between risk factor conditions and age-related brain diseases. Finally, in **chapter 7** we end with a general discussion of this thesis, overviewing the strengths and limitations of the thesis, the main findings and implications that arise from it, and suggestions for future research.

Of note: whereas Lcn2 describes the mouse form, the human form of this protein is usually referred to as neutrophil gelatinase-associated lipocalin (NGAL). For consistency, we will use the term Lcn2 throughout this thesis, except for the chapters in which specifically the human protein (NGAL) was studied (Chapters 2 & 5).

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